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Production of Metallothionein Polyclonal Antibodies Using Chickens as Model

Angélica María Ortiz-Bueno · Bertha Alicia León-Chávez · Alejandro Ruiz-Tagle · Patricia Lozano-Zarain · Leopoldo Castro-Caballero · William E. Achanzar · Eduardo Brambila

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Abstract The production of polyclonal antibodies (pAbs) against metallothioneins (MT) has been done in mammals. In this work, we describe a model where pAbs against rat liver MT were produced in chickens. Liver MT-1 and MT-2 isoforms isolated from rats were used as immunogens. MT was purified by exclusion chromatography and MT isoforms isolated by ionic exchange chromatography. Chickens were immunized with each isoform emulsified with Freund adjuvant over 6 weeks. MT-pAbs obtained from egg yolk were purified by ammonium sulfate precipitation followed by thiophilic interaction chromatography. MT-pAbs were characterized by ELISA, SDS-PAGE electrophoresis, and Western blot assays. Results showed significant titers (1:1,000) of MT-1 and MT-2 IgY in the eggs collected 30 days after the first immunization as determined by a direct ELISA assay; results also show a cross-reaction between MT-1 and MT-2 isoforms: however, the Abs obtained did not react with other non-MT proteins in hepatic homogenates. Sensitivity assays showed that MT-pAbs detected MT-1 and MT-2 at nanogram levels. These data suggest that chickens are an alternative model for producing pAbs against mammal high-homology proteins such as MT.

Keywords Chicken antibodies · Metallothionein · Metallothionein isoforms · IgY production

A. M. Ortiz-Bueno · B. A. León-Chávez · A. Ruiz-Tagle · L. Castro-Caballero · E. Brambila
Laboratorio de Investigaciones Químico Clínicas, Facultad de Ciencias Químicas,
Benemérita Universidad Autónoma de Puebla, Puebla, Puebla, México

P. Lozano-Zarain
Centro de Investigaciones en Ciencias Microbiológicas. Instituto de Ciencias,
Benemérita Universidad Autónoma de Puebla, Puebla, Puebla, México

W. E. Achanzar
Department of Toxicology, Bristol-Myers Squibb Pharmaceutical Research Institute, New Brunswick, NJ, USA

E. Brambila (✉)
Facultad de Ciencias Químicas, BUAP. Gral. Pedro Hinojosa 17, Lomas de Loreto, Puebla,
Puebla 72260, México
e-mail: ebrambil@siu.buap.mx

Introduction

Metallothionein (MT) is a multifunctional low molecular weight intracellular metal-binding protein characterized by a high content of cysteine residues and no aromatic amino acids or histidine [1, 2]. Structurally, MT is a highly conserved protein with the number and position of the cysteine residues being invariable, and containing Cys-X-Cys, Cys-X-Y-Cys, and Cys-Cys sequences in mammals [3, 4]. The primary biological role of MTs is not fully understood; proposed functions include sequestration of environmental toxic metals such as Cd, and Hg, regulation of essential metals such as Zn and Cu, Zn transference to apo-proteins and apo-enzymes, and free radical hydroxyl scavenging [5–9]. There have been a number of reports describing production of anti-MT polyclonal antibodies (pAbs) in small rodents, and their subsequent use as reagents to quantify MT concentrations by RIA and ELISA methods in biological fluids [10–18] and to identify MT by immunobloting and immunocytochemical localization [19–21]. However, there are several disadvantages to producing MT-pAbs in mammals. First, due to the poor inherent immunogenicity of MT, polymerization, or covalent coupling to an immunogenic moiety, such as keyhole limpet hemocyanin, is required. In addition, using mammals for antibody production requires large amounts of antigen and several months of antigen injections and animal bleeding [13, 22].

Birds are known to produce pAbs against highly conserved proteins of mammals [23–25] and the use of chicken egg yolk has been described as an alternative source of pAbs (IgY) [26], including the production of IgY against MT isoforms which were used to develop an ELISA assay [27]. The major advantage of raising pAbs in chickens is that conserved mammalian proteins are usually more immunogenic in phylogenetically distant birds, resulting in higher yields of IgY in comparison with the amount of IgG produced in mammals [26, 28]. Therefore, in this work, we described the production of pAbs against MT-1 and MT-2 isoforms, in chickens as an alternative.

Materials and Methods

Animals and Treatments

Adult Wistar rats (from the University of Puebla animal breeding facility) weighing 150 to 200 g were housed in polycarbonate cages with sawdust bedding and kept at controlled conditions (temperature of 21±2°C; 12 h light cycle starting at 0600; and 50% relative humidity). The rats were given food (Purina chow, Mexico) and water *ad libitum*. To obtain maximum yielding of hepatic MT, rats received an intraperitoneal injection of 1 mg/kg $CdCl_2$, and, 24 h later, 3 mg/kg of $CdCl_2$.

Inbred White Leghorn hens (28 weeks, SPF status, ALPES, Mexico) were housed in 50×50 cm individual cages with nest boxes in a standard animal room with 14/10 h light/dark cycle. The hens were fed with a laying hen diet (Purina) and water ad libitum.

Metallothionein Isoforms Isolation

MT-1 and MT-2 isoforms from rat liver were purified as described previously [29]. Briefly, rats with $CdCl_2$-induced hepatic MT were euthanized by exsanguinations, and immediately after, livers were excised and thoroughly perfused with ice cold saline (0.9% NaCl); then cut in small pieces and homogenized in four volumes of 0.25 M sucrose, 1 mM dithiotreitol, and 0.1 M Tris–HCl–acetate buffer, pH 8.6, containing 0.1% sodium azide,

using a Potter-Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 18,000×*g* for 30 min at 4°C, and the supernatant concentrated to 20 mL using a YM3 membrane (Amicon, Millipore, México). The concentrate was placed in Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ) chromatographic column previously equilibrated with the Tris–HCl–acetate buffer, pH 8.6. Samples were eluted using the same buffer, and the fractions (4.5 mL) containing MT were pooled and applied to a DEAE Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with the above buffer. Loading was accomplished at 0.3 mL/min flow rate, then a 2 to 250 mM Tris–acetate, pH 8.6, linear gradient was used. Fractions (6.0 mL) were collected and the MT isoforms present in each fraction were identified. The MT-1-contained fractions were pooled, as well the MT-2-containing fractions. Identification of MT in the fractions obtained from exclusion and ionic exchange chromatography was accomplished by measuring Cd–MT complex absorption maximum at 254 nm and Cd content by atomic absorption spectrophotometry. MT isoforms purity was assessed by SDS-PAGE electrophoresis [30] and gel silver staining (Bio-Rad, Hercules, CA).

Immunization

Two laying hens were injected at each of four different sites on the back with 100 μg of MT-1 or MT-2 in 250 μL Tris–HCl 0.01 M, pH 8.6 emulsified with an equal volume of complete Freund's adjuvant. The hens were boosted two times, 2 and 4 weeks later, with 100 μg of MT-1 or MT-2 in incomplete Freund's adjuvant.

Antibodies Extraction and Purification

Eggs were collected daily, marked and stored at 4°C until individual processing. Yolks were separated from the whites using an egg separator and washed several times with deionized water, collected in a graduated cylinder, diluted 1:10 with deionized water, and frozen for 24 h at −20°C. After this time, each egg yolk suspension was unfrozen until it reached room temperature and then centrifuged at 4,500×*g* for 45 min. Pellet was discarded and supernatant filtered through cotton wool and concentrated to 15 mL with an YM30 membrane (Amicon). IgY in the concentrate was precipitated by the addition of 25% (*w*/*v*) ammonium sulfate and incubated by 20 min at room temperature and centrifuged for 30 min at 4,000×*g*, then, 40% (*w*/*v*) ammonium sulfate was added to the supernatants and incubated by 20 min at room temperature and, centrifuged for 30 min at 4,000×*g*. Pellet was dissolved in 2 mL PBS (10 mM sodium phosphate buffer, pH 7.0, containing 14 mM NaCl and 0.02% sodium azide) and dialyzed against PBS overnight at 4°C.

IgY was purified using thiophilic interaction chromatography (HiTrap IgY purification columns. Amersham Biosciences, Roosendahl, The Netherlands). The columns were equilibrated with 20 mM sodium phosphate, 0.5 M potassium sulfate, pH 7.5 (binding buffer). The dissolved 40% ammonium sulfate pellets were applied to the columns at a rate of 1.0 mL/min. Adsorbed IgY was eluted from the columns into 5.0-mL fractions using 20 mM sodium phosphate, pH 7.5 (elution buffer). After IgY elution, the columns were regenerated by washing with 20 mM sodium phosphate and 30% isopropanol, pH 7.5 (cleaning buffer), to remove bound lipids. IgY in the collected fractions was monitored by the absorption at 280 nm and pooled. IgY isolation and purification was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels.

IgY Titration

Anti-MT-1 and MT-2 titers were assayed using a direct ELISA test. Wells of Nunc-immuno plates were coated with 1 μg of MT-1 or MT-2 dissolved in 100 μL of 0.1 M carbonate buffer and the plate incubated for 18 h at 4°C. Nonspecific sites were blocked with PBS, 50 g/L BSA, IgG free, and 3 mmol/L NaN_3. After 30 min, excess of BSA was removed with PBS-Tween (0.1%) and 1:100, 1:200, 1:500, and 1:1,000 dilutions of IgY anti-MT-1 or MT-2 were added, and incubated for 1 h at room temperature. Plates were washed three times with PBS, and a horseradish-peroxidase-conjugated chicken anti-MT IgY (Anti-Chicken IgG, Sigma Chemical Co. St. Louis) was added (1:1,000) and incubated for 1 h at room temperature. The antibody–antigen complex was revealed by adding 100 μL of 2,2′-azine-bis(3 ethylbenzyl-thiazoline-6-sulfonic) acid (ABTS) containing 0.3% H_2O_2 into each well. After 15 min, optical density (O.D.) was determined at 415 nm using a Benchmark multiplate reader (Bio-Rad, Hercules, CA, USA) [31].

Antibodies Specificity and Sensitivity

IgY anti-MT specificity and sensitivity were determined with SDS-PAGE and immunobloting. SDS-PAGE was performed under non-reducing conditions using 18% polyacrylamide gels. MT-1, MT-2 (1 μg/well) were electrophoresed, then, proteins were transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences, Roosendahl, The Netherlands) using a

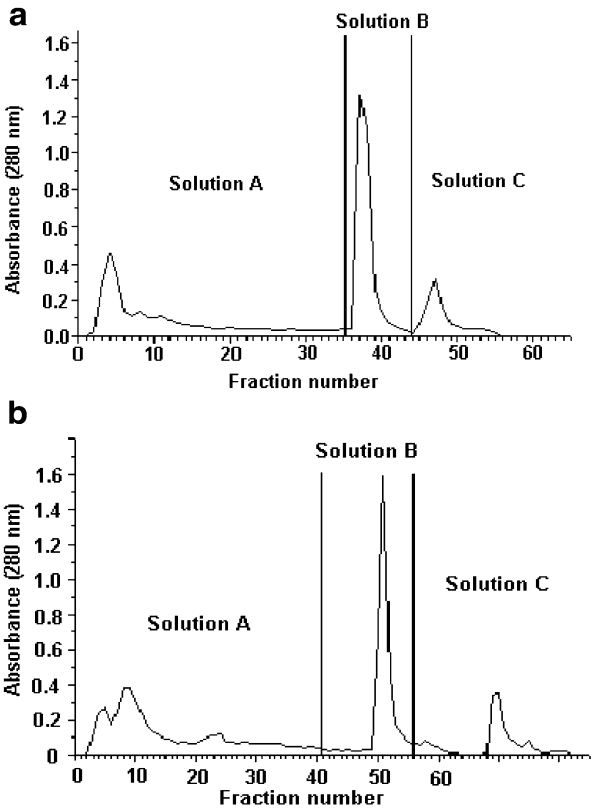

Fig. 1 Chicken antibodies purified by thiophilic interaction chromatography using a Hi-Trap affinity column. Antibodies were eluted using 20 mM sodium phosphate, 0.5 M potassium sulfate, pH 7.5 (binding buffer, *solution A*); 20 mM sodium phosphate, pH 7.5 (elution buffer, *solution B*) and 20 mM sodium phosphate and 30% isopropanol, pH 7.5 (cleaning buffer, *solution C*). **a** Anti-MT-1 IgY. **b** Anti-MT-2 IgY

Mini-PROTEAN 3 Electrophoresis Module and Mini Trans-Blot Module according to the manufacturer's directions (Bio-Rad, Hercules, CA). Protein transfer was confirmed by staining the membranes with Ponceau S. The membranes were blocked with 3% milk in PBS-T overnight at 4°C and incubated with several dilutions (1:200 to 1:2,000) of IgY anti-MT-1 or MT-2 for 1 h at room temperature. The membranes were washed with PBS-T, then incubated with a rabbit anti-chicken IgG conjugated with peroxidase (Sigma Chemical Co. St. Louis, MO) diluted 1:1,000 in PBS. A solution of 3% H_2O_2 and 0.5% diaminobenzidine in PBS was used as a substrate to visualize bound Abs [32]. Additional experiments were performed using rat hepatic homogenates; soluble proteins in concentration of 50 μg/well were electrophoresed on an 18% polyacrylamide gel, transferred to nitrocellulose membranes and immunoblotted with MT-1 and MT-2 IgY as described previously.

Results

Metallothionein Isoforms and Chicken Antibodies Purification

The MT isoforms, which were used for the production of anti-MT-1 and MT-2 IgY, were isolated from liver of rats treated with $CdCl_2$. MT purification was achieved by a well-known procedure described earlier [29]. MT isoforms were immunoblotted with commercial anti-MT monoclonal antibodies (Dako, Carpinteria, CA) to confirm their identity (data not shown).

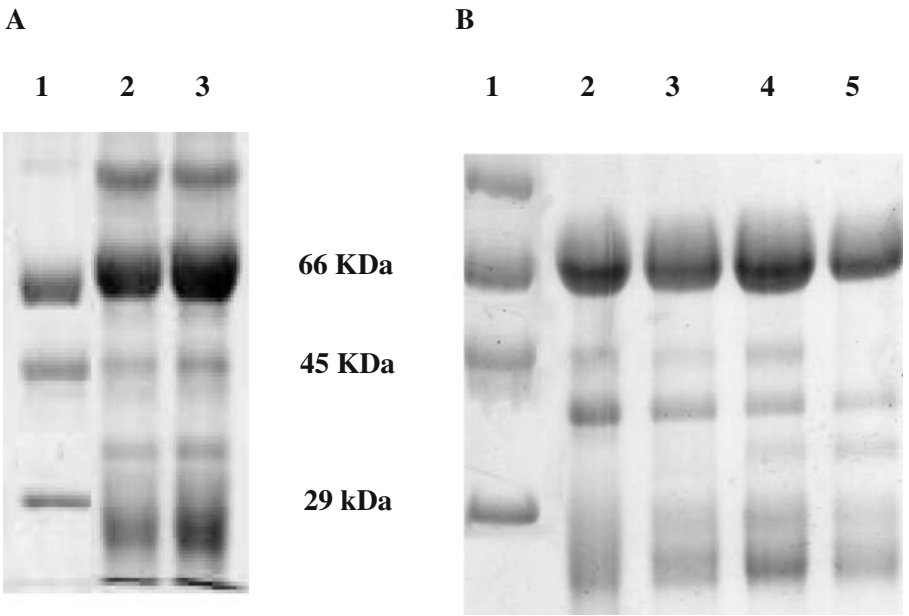

Fig. 2 Electrophoresis of anti MT-1 and MT-2-IgY isolated from egg yolk after ammonium sulfate precipitation and thiophilic interaction chromatography. Proteins were analyzed on a 12% SDS-polyacrylamide gel. **a** *Lane 1*, molecular mass markers, *lanes 2* and *3*, IgY isolated from chickens immunized with MT-1 and MT-2, respectively. **b** *Lane 1*, molecular mass markers, *lanes 2* to *5*, IgY isolated from two chickens immunized with MT-1 and two chickens immunized with MT-2

After chicken immunization, egg yolk anti-MT-1 and MT-2 IgY pAbs isolation and purification was achieved by ammonium sulfate precipitation and thiophilic interaction chromatography. Figure 1a shows the purification of anti-MT-1 IgY which eluted between a peak of non-bounded proteins and a peak of lipids. A similar elution profile was observed for anti-MT-2 IgY (Fig. 1b). Electrophoresis of the samples after ammonium sulfate precipitation confirmed the presence of IgY in samples of chickens immunized with either MT-1 or MT-2 (Fig. 2a). In both cases there are bands corresponding to IgY heavy (66 kDa) and light chains (28 kDa), in addition, several protein bands co-precipitate with IgY. Figure 2b shows the electrophoretic IgY pattern after thiophilic interaction chromatography: with lanes 2 and 3 containing the IgY anti-MT-1 IgY, and lines 4 and 5 containing anti-MT-2 IgY. Comparisons between Fig. 2a and b indicate that many of the non-IgY proteins present after ammonium sulfate precipitation were removed after thiophilic interaction chromatography.

IgY Titration

MT-1 IgY obtained from the eggs collected 30 days after the first immunization showed significantly titers at 1:100, 1:200, 1:500, and 1:1,000 IgY dilutions, as determined by a direct ELISA assay using 1 μg of MT-1 or MT-2 (Fig. 3a). Similar results were obtained when we assayed MT-2 IgY against the two MT isoforms; however, the final dilution of

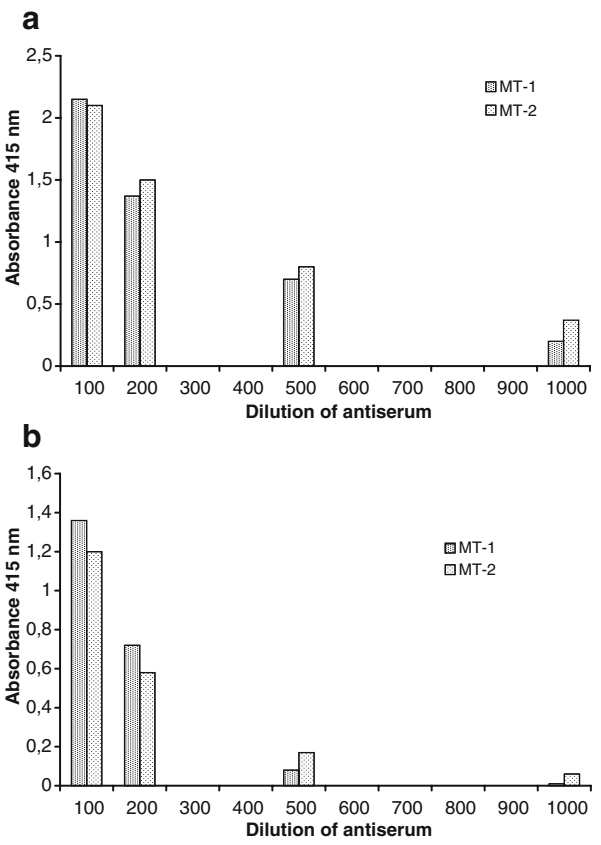

Fig. 3 Titration for anti-MT-1 and anti-MT-2 IgY. Dilutions of anti-MT-1 IgY (**a**) and anti-MT-2 IgY (**b**), isolated after 30 days of the first immunization to chickens, were allowed to react with 1 μg of MT-1 or MT-2 and then assayed with a direct ELISA

MT-2 IgY to detect MT-1 and MT-2 was 1:500 and 1:1,000, respectively (Fig. 3b). Results also showed that MT-1 and MT-2 IgY antibodies had cross reactivity with the two MT isoforms.

IgY anti-MT-1 and MT-2 Specificity

Immunoblotting was used to confirm that anti-MT-1 and MT-2 IgY obtained from immunized chickens were specific for hepatic MT-1 and MT-2, and did not recognize other

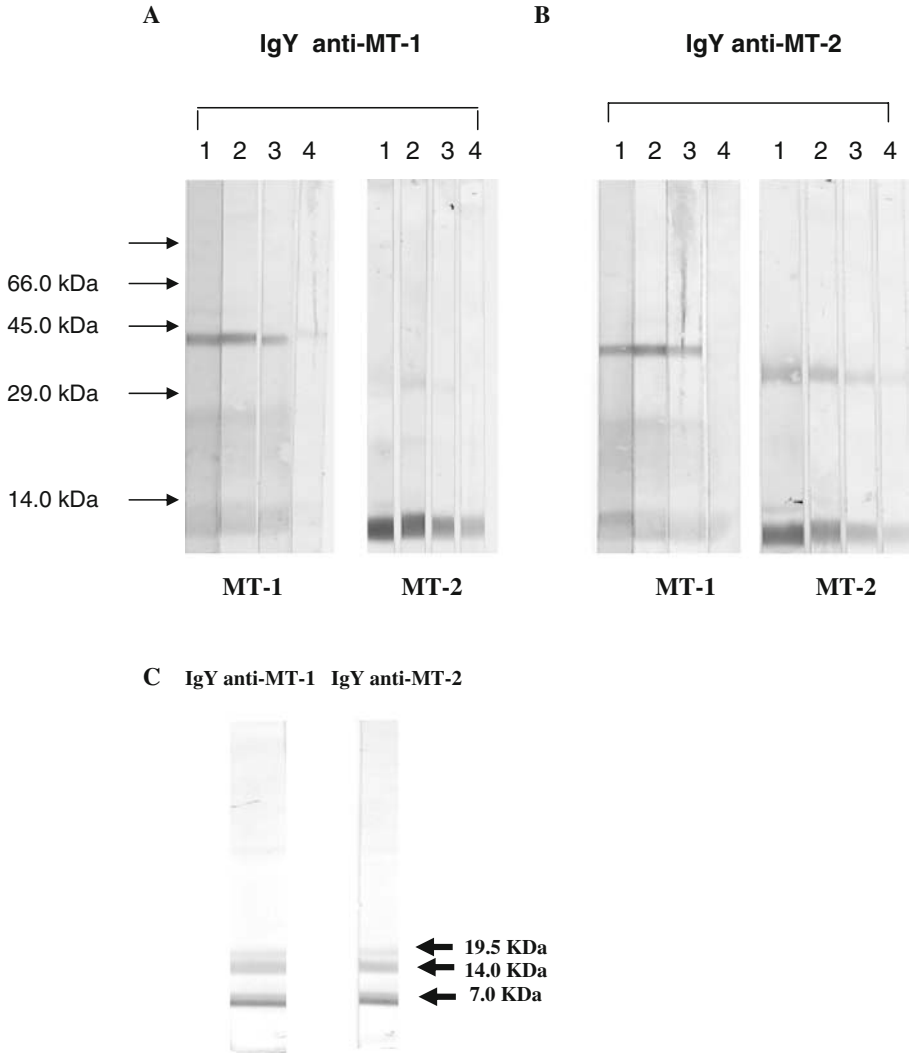

Fig. 4 Western blot analysis of purified MT-1, MT-2, and hepatic rat homogenate. **a** Western blot analysis of MT-1 and MT-2. *Lanes 1* to *4* correspond to 1:200, 1:500, 1:1,000, and 1:2,000 anti-MT-1 dilutions. **b** *Lanes 1* to *4* correspond to 1:200, 1:500, 1:1,000, and 1:2,000 anti-MT-2 dilutions. **c** Electrophoresis was performed in 18% SDS-PAGE Tris–HCl buffer, and proteins transferred to nitrocellulose membrane. Membranes were incubated with several dilutions of IgY anti-MT-1 or MT-2 antibodies. Anti-Chicken IgY-peroxidase-conjugated (1:1,000) and DAB (0.05%) were used for immunodetection (**a**)

non-MT hepatic proteins. Anti-MT-1 and MT-2 IgY reactivity was observed with both MT isoforms; when anti-MT-1 IgY was used as primary antibody, MT-1 was separated as a 42 kDa band, Western blot analysis also showed two additional bands with less staining intensity at 21 and 7 kDa, MT-2 was separated mainly as a monomer (7 kDa). Same results were obtained with MT-1 when anti MT-2 IgY was used as primary antibody, whereas, MT-2 reacted with this antibody giving bands at 35 and 7 kDa (Fig. 4a and b).

In other experiments, a hepatic rat homogenate was electrophoresed, proteins transferred to nitrocellulose, and immunoblotted with anti-MT-1 or MT-2 IgY. Figure 4c shows that anti-MT-1 and MT-2 IgY react with MT isoforms producing 19, 14, and 7 kDa bands, corresponding to MT polymeric and monomer forms.

IgY anti-MT-1 and MT-2 Sensitivity

IgY anti-MT sensitivity was evaluated against several MT-1 and MT-2 protein concentrations. Western blot analysis shows that anti-MT-1 Ig Y had reactivity against MT-1 at levels of 50 ng (Fig. 5a), whereas IgY anti-MT-2 had reactivity with MT-2 at levels of 100 ng (Fig. 5b).

Discussion

A number of methods have been used to quantify MT in biological fluids and tissues. They include assays that evaluate metal content in MT molecule, e.g., metal saturation assays, and those that detect and quantify MT protein, e.g., immunological methods [33]. The high sensitivity of Western blotting, RIA, and ELISA make these methods of choice to assay MT in biological fluids [12, 33]. Generally, the production of polyclonal antibodies to quantify MT by immunological methods has been done in mammals, however, the major disadvantages of using animals are the requirements of MT polymerization or coupling with other reagents to increase its immunogenicity, large amounts of antigen and long periods of immunization [10, 13]. Since the MT molecule has a high homology between

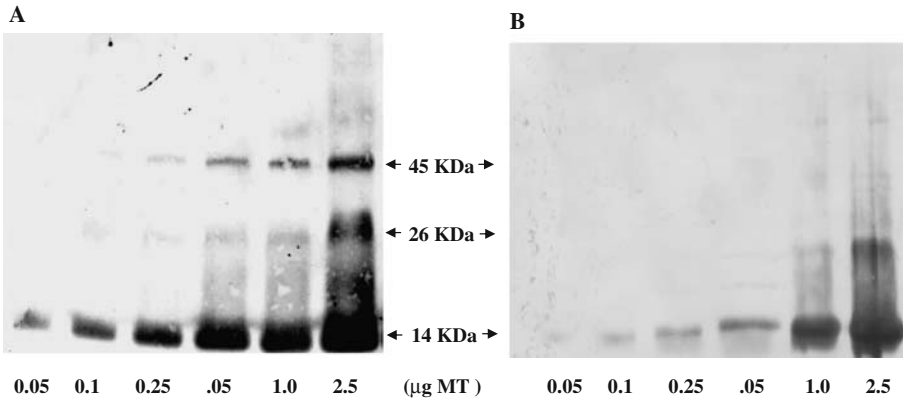

Fig. 5 MT antibodies' sensibility against MT-1 and MT-2 isoforms. Electrophoresis of MT-1 or MT-2 (0.05 to 2.5 µg) was performed in 18% SDS-PAGE, and proteins transferred to nitrocellulose membranes. Membranes were incubated with IgY anti-MT-1 (**a**) or anti-MT-2 (**b**) antibodies (1:200, dilution). Anti-Chicken IgY-peroxidase-conjugated (1:1,000) and DAB (0.05%) were used for immunodetection

mammals, in this work, we used hens as an alternative model for producing anti-MT pABs since they represent a phylogenetically distant species from mammals. In this work, hepatic MT isoforms were purified by chromatographic methods from rats previously injected with $CdCl_2$ to induce hepatic MT synthesis [33]. The individually purified MT-1 and MT-2 isoforms were then used to immunize hens according to the schedule described previously. IgY antibodies were purified from egg yolks by ammonium sulfate precipitation followed by thiophilic interaction chromatography. SDS-PAGE electrophoresis of the purified IgYs showed two distinct bands corresponding to the heavy and light IgY chains, comparison between IgY obtained after ammonium sulfate precipitation and thiophilic chromatographic separation show that several contaminant proteins were removed, however, some minor bands are present which likely represent incompletely reduced IgY molecules [34]. After 30 days of immunization with MT-1 or MT-2 (300 μg/hen), hens produced a significant amount (1:1,000 titer) of anti-MT-1 and MT-2 IgY, as shown by an ELISA assay (Fig. 3). These results contrast with the reports of other works, which show that the production of MT antibodies in mammals require longer immunization periods, several animals to obtain one animal with suitable antibody response and the use of high amount (mg) of antigen [12, 35, 36].

Chicken anti-MT-1 and MT-2 pAbs were not isoform specific as each Ab recognized both MT-1 and MT-2 isoforms. Western blot analysis show that MT-1 IgY react with purified MT isoforms producing a 42 kDa band, and two additional bands with lesser staining intensity at 21 and 7 kDa for MT-1, immunoblot also detected a 7 kDa band to MT-2 (Fig. 4a). MT-2 IgY reacted with both MT isoforms, MT-1 exhibited the same pattern as the experiments with MT-1 IgY, whereas, MT-2 showed additional bands at 35 and 7 kDa (Fig. 4b). Similar results have been reported by other researchers [17, 37]. Immunoblot of a hepatic rat homogenate shows that MT-1 and MT-2 reacted with anti-MT-1 and MT-2 IgY yielding 19, 14, and 7 kDa protein bands, corresponding to polymeric and monomer forms of MT, polymeric forms of MT has been previously reported to occur during the purification process [37, 38] or storage [39], monomers of MT-1 and MT-2 are thought to be oxidized to give polymerized MT-1 and MT-2. Zangger et al. (2001) have shown that these aggregates can be the result of intermolecular disulphide bonds between cysteines in the MT molecule [40]. Chicken anti-MT-1 and MT-2 antibodies exhibited a good sensitivity as 50 and 100 ng of MT-1 and MT-2 isoforms, respectively, could be detected by Western blot.

In conclusion, chickens are a good alternative to raise anti-MT pAbs. The production of IgY is fast and simple, require a relatively small amount of MT to produce an efficient immune response in short time, and demonstrate a good alternative to obtain antibodies against highly conserved mammal antigens such as MT.

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